

# EXHIBIT 3

# Identification of a Mononucleotide Repeat as a Major Target for Mitochondrial DNA Alterations in Human Tumors<sup>1</sup>

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## Abstract

Mitochondrial DNA (mtDNA) mutations scattered through coding and noncoding regions have been reported in cancer. The mechanisms that generate such mutations and the importance of mtDNA mutations in tumor development are still not clear. Here we present the identification of a specific and highly polymorphic homopolymeric C stretch (D310), located within the displacement (D) loop, as a mutational hotspot in primary tumors. Twenty-two % of the 247 primary tumors analyzed harbored somatic deletions/insertions at this mononucleotide repeat. Moreover, these alterations were also present in head and neck preneoplastic lesions. We further characterized the D310 variants that appeared in the lung and head and neck tumors. Most of the somatic alterations found in tumors showed deletion/insertions of 1- or 2-bp generating D310 variants identical to constitutive polymorphisms described previously. Sequencing analysis of individual clones from lymphocytes revealed that patients with D310 mutations in the tumors had statistically significant higher levels of D310 heteroplasmy (more than one length variant) in the lymphocyte mtDNA as compared with the patients without D310 mutations in the tumor mtDNA. On the basis of our observations, we propose a model in which D310 alterations are already present in normal cells and achieve homoplasmy in the tumor through a restriction/amplification event attributable to random genetic drift and clonal expansion.

## Introduction

Human mtDNA<sup>4</sup> is composed of a 16.6-kb, double-stranded, closed-circular DNA molecule (1). To date many common polymorphisms have been described in the mtDNA,<sup>5</sup> and most of them accumulate in the regulatory region or D-loop (2). In addition to neutral polymorphisms, mtDNA mutations have also been detected (3), many of them associated with some degenerative diseases, such as Leber's hereditary optic neuropathy (4), chronic progressive external ophthalmoplegia (5), and the mitochondrial encephalomyopathy, lactic acidosis and stroke episodes (6). Moreover, it has been reported that mtDNA mutations accumulate with age. Individuals over the age of 50 showed a higher frequency of somatic mtDNA mutations or

rearrangements in some tissues, such as brain and skeletal muscle, compared with younger individuals (7). The origin of somatic mtDNA mutations has been mainly attributed to oxidative damage from ROS that accumulate in postmitotic tissues with age or to deficient mtDNA repair mechanisms (8, 9).

Recently, we and others identified a high frequency of missense and frameshift mutations in mtDNA from primary human neoplasms (10-15). These mutations were scattered throughout many coding and noncoding regions of the mtDNA of the various tumors studied. The majority of somatic mutations in coding regions produced no amino acid changes, but those resulting in coding changes were generally confined to respiratory complex I (11-15).

To further define the pattern of mtDNA mutations in lung tumors, we initially sequenced two large regions in 27 primary tumors. Interestingly, we found that a specific mononucleotide repeat (D310) was frequently altered in tumors as compared with the matched normal mtDNA. To evaluate the frequency of D310 alterations in cancer, we screened a large panel of primary tumors and some preneoplastic lesions. On the basis of our data, we propose a model to explain the origin of these specific homoplasmic alterations in primary human cancers.

## Materials and Methods

**Patient Samples and DNA Extraction.** Primary tumors and matched normal tissue from lymphocytes were obtained from 247 patients including 100 lung tumors, 51 HNSCC, 8 gastric tumors, 17 breast tumors, 25 colorectal tumors, 15 bladder tumors, 15 ovarian tumors, and 16 prostate tumors as well as 14 hyperplastic and dysplastic lesions from 10 head and neck cancer patients. Informed consent was obtained from each patient. Tumor DNA was microdissected to obtain at least 70% of tumor cells. DNA from normal and tumor tissue was extracted as described previously (13).

**Sequencing of the mtDNA.** Two different independent fragments of 2466 and 2607 bp (13) were PCR amplified and manually sequenced in 27 primary lung tumors. The first fragment contains the noncoding D-loop region, and the second fragment contains the *ND4* (NADH subunit 4), *ND4L* (NADH subunit 4 L), *ND3* (NADH subunit 3) genes and the tRNAs *tRNA<sup>Arg</sup>*, *tRNA<sup>His</sup>*, *tRNA<sup>Ser</sup>*, and *tRNA<sup>Leu</sup>*. For DNA amplification, total DNA was subjected to step-down PCR protocol: 95°C for 2 min, 1 cycle; 95°C for 30 s, 64°C for 1 min, 70°C for 2 min 30 s, three cycles; 95°C for 30 s, 58°C for 1 min, 70°C for 2 min 30 s, 2 cycles; 95°C, for 30 s, 57°C for 1 min, 70°C 2 min 30 s, 35 cycles; and a final extension at 70°C for 5 min. PCR products were excised from a 2% agarose gel and purified with Qiagen gel extraction kit (Qiaquick columns; Qiagen, Chatsworth, CA). Cycle sequencing was performed according to the manufacturer's instructions (Perkin-Elmer, Roche Molecular Systems, Inc. Branchburg, NJ). Because some of the previously reported mtDNA mutations are identical to polymorphisms and some mutations may be undistinguished from rare polymorphisms, tumor DNA and paired blood samples were analyzed in all cases. Analysis in those samples showing mutations was repeated in two independent PCR and sequencing reactions.

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<sup>4</sup> The abbreviations used are: mtDNA, mitochondrial DNA; HNSCC, head and neck squamous cell carcinoma; D310, polytract of cytosines at nucleotide 310 of the mitochondrial genome; D-loop, displacement loop; ROS, reactive oxygen species; CSBI and CSBI, conserved sequence block I and II, respectively.

<sup>5</sup> Internet address: <http://infinity.gen.emory.edu/mitomap.html>.

**Genotyping Assays of the D310 repeat.** Between 10 and 100 ng of total DNA were used to amplify the D310 repeat from paired normal and tumor samples. The sequence for the forward primer was 5'-ACAATTGAATGTCT-GCACAGCCACTT-3' and for the reverse primer 5'-GGCAGAGATGTGTT-TAAGTGCTG-3'. The concentrations of the reagents used in the PCR were as described previously (16). After an initial denaturalization step at 95°C for 2 min, samples were cycled 35 times as follows: 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The final extension was at 72°C for 4 min. One-tenth of the PCR product was loaded in the 6% denaturing polyacrylamide gel, as described (17).

To rule out possible mistakes in the PCR reaction, the assay was repeated for all normal-tumor pairs that showed D310 alterations in the tumor DNA. We also confirmed that normal and tumor belonged to the same individual by amplifying at least one highly polymorphic microsatellite marker from the nuclear DNA. This is especially important because the constitutive polymorphisms described for the D310 tract are identical to the most common alterations found in the mtDNA from tumor tissues.

**Cloning of the D310 Mononucleotide Repeat.** A 346-bp fragment containing the D310 repeat was PCR amplified from lymphocyte DNA of eight patients. The sequence of the primers was: forward, 5'-ACAATTGAAT-GTCTGCACAGCCACTT-3'; and reverse, 5'-TGTGGGGGGTGTCTTT-GGGG-3'. The PCR products were purified with phenol-chloroform, followed by ethanol precipitation. Afterward, the PCR products were cloned into a TA-PCR cloning vector (Invitrogen). Twenty colonies of each PCR product were selected for further growth. DNA was extracted and manually sequenced (Perkin-Elmer, Roche Molecular Systems, Inc. Branchburg, NJ) using the forward primer 5'-GGCAGAGATGTGTTTAAGTGCTG-3'.

## Results

**Sequencing Analysis of mtDNA in Lung Tumors.** We initially analyzed 27 primary lung tumors for mtDNA mutations, and a high number of germ-line polymorphisms were detected. Some of these polymorphisms had not been described previously and were submitted to the mtDNA database.<sup>5</sup> In addition to polymorphisms, somatic variants were found in nine of the tumors. Two of these tumors harbored a C-to-G transversion in nucleotide 16,114 (inside the D-loop) and an A-to-G transition in position 10,448 (inside *tRNA<sup>Arg</sup>*), respectively. Remarkably, the remaining seven tumors showed deletions or insertions in a mononucleotide repeat sequence (CCCC...CCCTCCCCC) between nucleotides 303 and 316–318 (inside the D-loop). For easier designation, we will refer to this region as D310, a term coined previously by other investigators (18). D310 is a highly polymorphic tract, and the number of cytosines in the first stretch can vary from at least 7-C to 9-C among normal individuals (Fig. 1A; Refs. 18, 19). Because a copy of part of the mitochondrial D-loop sequence has been detected in the nuclear genome (GenBank accession number AK000414), we resequenced eight random samples using the larger 2.5-kb-specific mtDNA PCR product and confirmed that somatic changes occurred in the mtDNA (data not shown). Moreover, our primers did not generate PCR products in a human cell line devoid of mtDNA ( $p^0$ ).

**Genotyping of the D310 Repeat in Primary Tumors.** To determine whether these mtDNA alterations in the D310 repeat were present in other tumor types, we screened 220 additional primary neoplasms and their matching lymphocytes (total, 247 primary tumors) with a simple PCR assay that includes the D310 repeat (Fig. 1B). We detected somatic variations (D310 changes in the tumor with respect to the matched lymphocyte DNA) in 22% (55 of 247) of all tumors and in every tumor type analyzed except in ovarian and prostate cancer. We discarded the possibility that the D310 mutations were tissue-specific polymorphisms by analyzing the corresponding normal tissue of some of the tumors (normal breast tissue of the 17 breast cancers) and finding the same germ-line sequence identified in the DNA from matched lymphocytes (Fig. 1B). Sixty-nine % (38 of 55) of the alterations in the tumor DNA were insertions or deletions

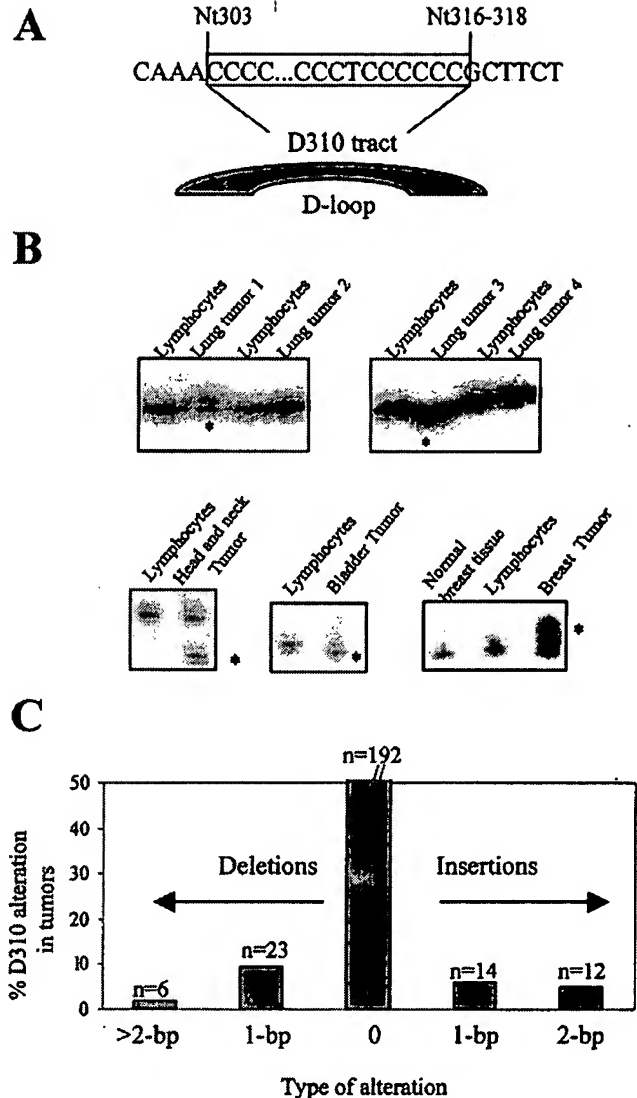


Fig. 1. Alterations in the D310 mononucleotide repeat. A, representation of the location and structure of the D310 repeat in the D-loop. B, PCR-based assay showing the insertions/deletions at the D310 repeat in several tumor types. \*, somatic alterations in the tumor DNA. The mtDNA variations are clearly present in the tumor but absent in normal lymphocytes or breast tissue. C, distribution of the D310 variants in all of the tumors. Sequenced variants were homoplasmic (or nearly homoplasmic) in all cases, as described previously (11, 13). The presence of normal bands in this nonquantitative assay may be attributable to minimal heteroplasmy or more likely to the presence of normal DNA contamination.

of 1 bp, and 31% (17 of 55) were changes of 2 bp or more (up to 8 bp; Table 1). Tabulation of individual D310 mutations in all tumors displayed a narrow normal distribution (Fig. 1C). Interestingly, we also observed the presence of D310 changes in 2 of the 14 hyperplastic or dysplastic lesions from 10 patients who eventually progressed to HNSCC, indicating that these abnormalities can occur early during tumor progression (Fig. 2A).

**Distribution of D310 Polymorphisms and D310 Alterations in Lung and HNSCC Patients.** To better understand the nature of the D310 variants, we compared the patterns and distribution of the alterations in lung and HNSCC patients. The presence of D310 alterations was significantly higher in the HNSCCs (19 of 51; 37%) compared with lung carcinomas (16 of 100; 16%;  $P = 0.006$ ; Fisher's exact test). Most of the mutations observed in the lung tumors were insertions or deletions of 1 bp (14 of 16; 87.5%). The remaining two

Table 1 Somatic deletions/insertions (1-bp or higher) in the D310 repeat in various tumor types

Tumor type	No. of alterations	1 bp	>1 bp
Gastric (n = 8)	5 (62.5%)	4	1
HNSCC (n = 51)	19 (37%)	11	8
Breast (n = 17)	5 (29%)	3	2
Colorectal (n = 25)	7 (28%)	4	3
Lung (n = 100)	16 (16%)	14	2
Bladder (n = 15)	3 (20%)	2	1
Ovarian (n = 15)	0		
Prostate (n = 16)	0		
Total (n = 247)	55 (22%)	38 (69%) <sup>a</sup>	17 (31%) <sup>a</sup>

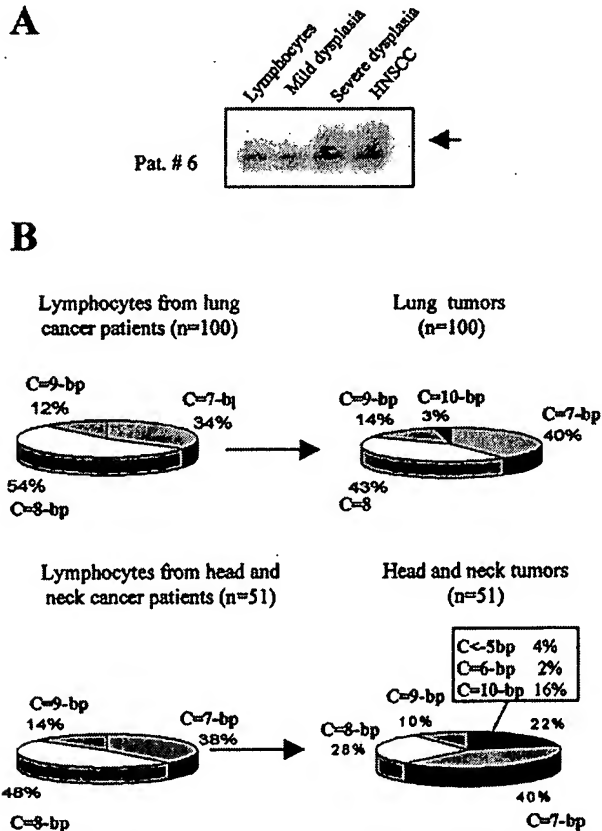
<sup>a</sup> Percentages calculated in relation to the total number of alterations.

Fig. 2. A, presence of D310 alterations in preneoplastic lesions from HNSCC patients. B, frequency and distribution D310 polymorphisms in lymphocytes and D310 alterations in tumor from HNSCC patients. In those cases with apparent heteroplasmy on denaturing gels, only the predominant variant was considered.

(2 of 16; 12.5%) were 2-bp insertions. In HNSCCs, 58% (11 of 19) harbored 1-bp deletion/insertions, and 32% (6 of 19) harbored 2-bp insertions. Two large deletions (5 and 7 bp) were observed in 2 of the tumors (2 of 19; 11%; Fig. 1B).

As shown in Fig. 2B, no significant differences between the frequency and distribution of the constitutive polymorphisms were observed in lymphocytes from patients with lung and HNSCC. In both groups, the most common variants were 8-C and 7-C (number of cytosines in the first stretch of the D310 tract), followed by 9-C. Then, we analyzed the spectrum of the genetic variants in lung and HNSCC and compared it with the distribution of the polymorphisms in lymphocytes. For lung tumors, we found that the genetic variants demonstrated nearly the same distribution as the polymorphisms observed in lymphocytes, except for three cases (3%) that harbored a new 10-C variant. Most of the genetic variants detected in the HNSCC also mirrored the range of constitutive polymorphisms. However, ~22%

of these variants were deletions/insertions not observed as constitutive polymorphisms, including the two large deletions that almost abrogate the first stretch of Cs in the D310 repeat.

For the lung cancer patients, we had clinical and pathological information available. The presence of D310 changes in the tumor mtDNA was not associated with the age, sex, race, or smoking history of the individuals nor with any histological types or genetic parameters (*p53*, *K-ras*, and *p16* alterations and chromosomal abnormalities) of the tumors (data not shown).

**Detection of Heteroplasmy at the D310 Repeat in Normal Lymphocytes from Cancer Patients.** We considered whether these alterations arise from the clonal expansion of normal cells, which already harbor a heteroplasmic polymorphism, or whether they are generated as new somatic variants during tumor development. To address this issue, we amplified a 350-bp fragment, containing the D310 repeat, from the lymphocyte DNA of eight cancer patients (four of them harboring D310 alterations and four without alterations in the primary tumor DNA) and subcloned the PCR products. Sequence analysis of 122 individual clones identified minor populations of D310 variants in the DNA from normal lymphocytes. However, the Poisson distribution of these clone populations was clearly different among individuals with and without apparent D310 alterations in the tumors. Length variations in the D310 were detected in 15 of the 61 (25%) clones from lymphocytes of patients with abnormalities in the tumor but only in 3 of the 61 (5%) clones from patients without D310 alterations in the tumor ( $P = 0.002$ ; Fisher's exact test). The D310 variations detected in the normal DNA included those observed in the matched tumor DNA, except for the 5-bp deletion present in tumor HN1164 (Table 2), which was not detected among the 16 clones from matched normal lymphocytes.

## Discussion

We have found that the D310 mononucleotide repeat in mtDNA is a hot spot for somatic deletions/insertions in many cancers. The D310 repeat is part of the CSBII located (in humans) 92 bp from the heavy strand replication origin. Together with the CSBI and CSBIII regions, CSBII contributes to the formation of a persistent RNA-DNA hybrid, also termed the R-loop, leading to the initiation of mtDNA replication (20, 21). It is not yet clear whether sequence variants of the D310 region would lead to alterations in mtDNA replication. Some severe alterations could lead to functional impairment of the mitochondria and somehow promote a growth advantage for the tumor cell.

We analyzed the presence of D310 changes in a large number of primary tumors of several origins and in hyperplastic and dysplastic lesions from head and neck cancer patients. Most of the alterations in

Table 2 Comparison between D310 heteroplasmic genetic variants in the lymphocytes: DNA from individuals with and without D310 alterations in the primary tumor

	No. of clones	D310 variants (lymphocyte DNA)
L738+ <sup>a,b</sup>	18	4
HN1164+	16	4
B41+	15	6
B42+	12	1
Total+	61	15 <sup>c</sup>
L788-	19	0
L1973-	14	1
B26-	15	2
B38-	13	0
Total-	61	3 <sup>c</sup>

<sup>a</sup> L, lung cancer; HN, head and neck cancer; B, breast cancer. The total + was statistically higher compared to total -.

<sup>b</sup> +, samples with D310 alterations in the tumor; -, samples without D310 alterations in the tumor.

<sup>c</sup>  $P = 0.002$ ; Fisher's exact test.

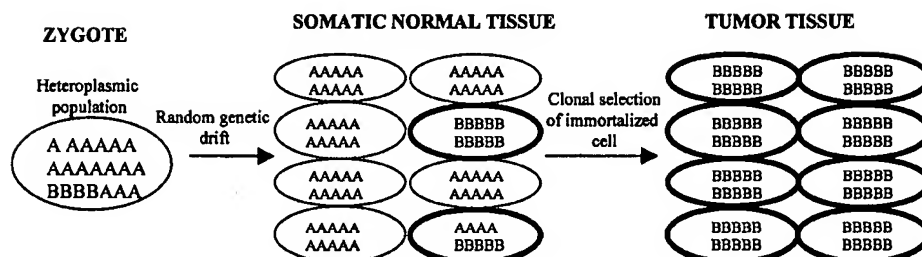


Fig. 3. Proposed model for the generation of neutral D310 homoplasmic alterations in tumor mtDNA. Unequal partitioning of genomes during cytokinesis can lead to differences between daughter cells in somatic tissue through stochastic segregation and random genetic drift. A, A/B, and B represent the mitochondria organelles harboring the predominant mitochondrial genomes A, B, or heteroplasmic (both A and B). After clonal selection of an immortalized cell attributable to nuclear oncogene mutations, tumor tissue harbors the homoplasmic B variant. Tumors may also achieve homoplasmicity from heteroplasmic A/B cells by recapitulating this process in conjunction with clonal expansion driven by additional nuclear mutations. Differences in observed frequencies of neutral polymorphism variants in various tumors types may be attributable to the number of clonal expansions preceding clinical presentation (30).

D310 we detected in the tumor DNA were 1-bp deletions/insertions, and almost all were in the polymorphic length range (between 7-C and 9-C; Refs. 22, 23). These observations suggest that most D310 variants in tumors are unlikely to lead to functional impairment of the mitochondria. A few tumors did show unusual variants (6-C and 10-C), which may represent rare polymorphisms or may originate during tumor progression. Moreover, at least two head and neck tumors harbored big deletions (5 and 7 bp), which may be mutations that originate during tumor development and may have some affect in the mitochondrial function.

Intriguingly, we also observed that patients with D310 alterations in tumor DNA showed higher levels of D310 heteroplasmy (more than one length variant) in lymphocyte DNA than patients without D310 alterations in the tumor mtDNA. Heteroplasmy, at specific mitochondrial genes, have been reported in mitochondrial diseases, presumably because homoplasmic mutations would be lethal (24), and in polymorphic sites such as D310 (18, 25). Rapid switching of apparently neutral polymorphisms has been observed through new generations (26) and in *Caenorhabditis elegans* development (27). To explain these observations, a bottleneck model has been proposed for the transmission of mtDNA. According to this hypothesis, a restriction/amplification event or "bottleneck" takes place in heteroplasmic individuals mainly during oocyte maturation. Through this restriction/amplification mechanism a single or small number of founder mtDNAs populate the organism (reviewed in Ref. 28). We propose that a similar restriction/amplification event occurs during tumorigenesis, resulting in the expansion of already present mitochondrial variants to homoplasmy in the tumor tissue. Different variants may already be present in a substantial minority of mitochondria from normal cells, either individual cells with different mitochondrial genotypes or different mitochondrial genotypes in the same cells. Cells that are homoplasmic for a given mtDNA variant may arise in normal tissues as a consequence of unequal partitioning of daughter mitochondria during cell division. In agreement with this hypothesis, normal cells that are homoplasmic for minor mtDNA variants have been detected in normal epithelial tissues (29). These changes would become dominant in the tumor mtDNA because of clonal expansion (driven by nuclear oncogene/tumor suppressor gene mutations) of the tumor progenitor cells that carries the minor mtDNA variants (Fig. 3). Further support for this model comes from the distribution of these variants, which demonstrates a reasonable likelihood of switching from one polymorphism to another without readily apparent bias. This model also suggests that highly polymorphic mitochondrial DNA variants may be useful as molecular clocks, adding insight into the nature of clonal expansions in tumor cell populations (30).

We did not observe any D310 variations in prostate or ovarian tumors, and the rates of D310 alterations were different among the

distinct tumor types analyzed. Moreover, some of our observations, such as the statistically significant different rates of D310 variations in lung tumors (16% in 100 tumors) and HNSCC (37% in 51 tumors), suggest the existence of alternative mechanisms for the generation of some D310 alterations, such as the rate of acquired mutations during tumor development, the number of mitochondrial organelles/cell, or the intrinsic differences in the number of cell divisions prior to tumor formation in different tissues (29). Although we present a model for clonal expansion of nonfunctional mtDNA variants, it is probable that true functional relevant mtDNA mutations also arise in human cancers: (a) cell fusion experiments suggest that particular mtDNA mutations are dominant, able to replace recipient mtDNA and achieve homoplasmy in tissue culture quite rapidly (11); (b) some of our observed D310 variants harbor large C-tract deletions that are likely to interfere with the initiation of mtDNA replication (20–22). One such 5-bp deletion in a HNSCC was not detected in the matched lymphocyte DNA of the affected patient; and (c) virtually all coding mutations described in human cancers are located in the respiratory complex I. Although coding mutations downstream of complex I may be selected against because of poor or absent energy production, their segregation in complex I suggests some functional significance to these mutations. For example, it is possible that increased ROS produced from complex I mutations may be mutagenic to mtDNA and even increase the mutation load in the nuclear genome of cancer cells. Moreover, *in vitro* experiments demonstrate that damage to DNA by ROS may target mutations within repetitive sequences (31, 32).

Clearly, the location and nature of somatic mtDNA variants in cancer must be considered in assessing their functional relevance. We present a compelling model for expansion of nonfunctional alterations, but we have no data on the rates at which switching occurs in the somatic tissue of normal individuals. It is possible that subtle repair deficiencies in tumor cells (or cancer-prone individuals) may increase the rate of switching and the frequency of heteroplasmy. Initial heteroplasmy may lead to homoplasmy of more functionally relevant variants in tumor cells through continued clonal evolution. These important questions suggest that the study of somatic mtDNA variants in cancer is ripe for further investigation.

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